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ARTICLE

Trophoblast attachment to the endometrial epithelium elicits compartment-specific transcriptional waves in an in-vitro model

BIOGRAPHY

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KEY MESSAGE

A model is presented describing the transcriptional events regulating early implantation in a temporal and compartment-specific manner, providing a source of candidate molecules involved in successful embryo attachment. This process is characterized by early and transient gene expression up-regulation in the epithelium and overall sustained gene up-regulation in the trophoblast.

ABSTRACT

Research question: Which are the early compartment-specific transcriptional responses of the trophoblast and the endometrial epithelium throughout early attachment during implantation?

Design: An endometrial epithelium proxy (cell line Ishikawa) was co-cultured with spheroids of a green fluorescent protein (GFP) expressing trophoblast cell line (JEG-3). After 0, 8 and 24 h of co-culture, the compartments were sorted by fluorescence-activated cell sorting; GFP+ (trophoblast), GFP– (epithelium) and non-co-cultured control populations were analysed (in triplicate) by RNA-seq and gene set enrichment analysis (GSEA).

Results: Trophoblast challenge induced a wave of transcriptional changes in the epithelium that resulted in 295 differentially regulated genes involving epithelial to mesenchymal transition (EMT), cell movement, apoptosis, hypoxia, inflammation, allograft rejection, myogenesis and cell signalling at 8 h. Interestingly, many of the enriched pathways were subsequently de-enriched by 24 h (i.e. EMT, cell movement, allograft rejection, myogenesis and cell signalling). In the trophoblast, the co-culture induced more transcriptional changes and regulation of a variety of pathways. A total of 1247 and 481 genes were differentially expressed after 8 h and from 8 to 24 h, respectively. Angiogenesis and hypoxia were over-represented at both stages, while EMT and cell signalling only were at 8 h; from 8 to 24 h, inflammation and oestrogen response were enriched, while proliferation was under-represented.

Conclusions: Successful attachment produced a series of dynamic changes in gene expression, characterized by an overall early and transient transcriptional up-regulation in the receptive epithelium, in contrast to a more dynamic transcriptional response in the trophoblast.

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KEYWORDS

Attachment
Endometrial receptivity
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INTRODUCTION

Implantation is a major bottleneck in human reproduction (*Polanski et al., 2014*). The average implantation rate for an embryo ranges from 30% to 40% (*Coughlan et al., 2014*). Recurrent implantation failure (RIF) is estimated to occur in approximately 4% of IVF cycles (*Koot et al., 2012*), although estimates vary because there are several somewhat different definitions of RIF in the literature. Implantation of the blastocyst in the receptive endometrium is a sequential process involving apposition, attachment and invasion that precedes the establishment of pregnancy (*Wang and Dey, 2006*). Successful implantation requires embryo competence and endometrial receptivity, both of which are dynamic and highly regulated states (*Wang and Dey, 2006*). In addition to genetic disorders (which are a major cause of implantation failure and miscarriage), embryo competence, quality and ultimately developmental potential depend on the embryo achieving the correct regulatory, signalling and metabolic states (*Fu et al., 2009*; *Hourvitz et al., 2006*; *Lundin et al., 2001*; *Simon and Laufer, 2012*; *Sjoblom et al., 2006*). A key determinant of these embryonic states is their underlying transcriptional dynamics; for instance, waves of embryonic transcriptional activation direct early development and the symmetry breaking needed for cell fate specification (*Shi et al., 2015*; *Vassena et al., 2011*). Endometrial receptivity, driven by ovarian steroids, results in a 'window of implantation' that is also dependent on the establishment of correct transcriptional signatures (*Diaz-Gimeno et al., 2011*; *Enciso et al., 2018*). During every menstrual cycle, the endometrium undergoes cyclic proliferation and differentiation. If a viable embryo is present, invasive placentation and pregnancy occur; in its absence, the endometrium undergoes breakdown and repair (*Evans et al., 2016*). The stromal compartment of the endometrium plays an essential role in the establishment and maintenance of pregnancy; upon decidualization, this compartment becomes an embryo quality biosensor (*Brosens et al., 2014*; *Teklenburg et al., 2010*). Disordered decidualization is linked to defective embryo selectivity, which may be responsible for the extended receptivity to, and non-selective acceptance of, low-quality embryos in women suffering recurrent pregnancy loss (RPL). Conversely, a decidua that is too restrictive leads to the rejection of high-quality embryos and implantation failure (*Salker et al., 2012*; *Weimar et al., 2012*).

A particular aspect of a competent blastocyst and a receptive endometrium required to support successful implantation is their ability to coordinate their behaviour. Asynchrony or faulty molecular cascades occurring during embryo–endometrium interactions may lead to implantation failure, therefore limiting IVF success rates (*Koot et al., 2016*; *Valdes et al., 2017*). The importance of the transcriptional regulation of trophoblast–decidual communication during early pregnancy and placentation has recently been reported (*Vento-Tormo et al., 2018*). However, knowledge about the mechanisms regulating the first physical interaction at the maternal–fetal interface, i.e. apposition and attachment of the blastocyst to the epithelial surface, is still limited. Recent studies have demonstrated that molecular signals from the luminal epithelium regulate the trophoblast differentiation needed for barrier breaching during attachment and that uterine glands coordinate the timing of implantation (*Kelleher et al., 2018*; *Ruane et al., 2017*). Previous studies have compared the individual transcriptional profiles of the preimplantation embryo and the receptive endometrium (*Altmae et al., 2017*; *Haouzi et al., 2011*). Nevertheless, the specific transcriptional dynamics of the trophoblast/epithelium cross-talk have not been examined in a compartment-specific manner.

This group has recently determined that the receptive epithelium mounts a transcriptional response to trophoblast challenge that is severely muted when the epithelium is non-receptive (*Vergaro et al., 2019*). Combining cell sorting and RNA-seq analysis, the transcriptional responses that the trophoblast (modelled by spheroids of the JEG-3 trophoblastic cell line) and the receptive epithelium (modelled by the Ishikawa endometrial epithelial cell line) undergo during the first 8 and 24 h of attachment *in vitro* have been characterized.

MATERIALS AND METHODS

Cell culture

The human endometrial adenocarcinoma cell line Ishikawa (European Collection of Authenticated Cell Cultures, Porton Down, Salisbury, UK; cat. no. 99040201) was cultured in minimum essential medium alpha modification (MEMα, nucleosides, no phenol red) containing 5% fetal bovine serum, 10 mmol/l of non-essential amino acids (MEM Non-Essential Amino Acids Solution) and 100 mg/ml streptomycin and 100 IU/ml penicillin (PenStrep). The human trophoblast choriocarcinoma cell line JEG-3 (American Type Culture Collection, Manassas, Virginia, USA; cat. no. HTB-36) was cultured in Dulbecco's modified Eagle medium (DMEM, high glucose, GlutaMAX™ supplement) supplemented with 10% fetal bovine serum, 1 mmol/l sodium pyruvate and 100 mg/ml streptomycin and 100 IU/ml penicillin (PenStrep). Cells were maintained at 37°C in 5% CO_2 and media changed every other day. Unless specified, all reagents were obtained from Gibco, Thermo Fisher Scientific, Waltham, Massachusetts, USA.

Fluorescent trophoblast spheroids

Fluorescent spheroids of the trophoblast cell line JEG-3 were generated by transduction with recombinant lentiviral particles expressing GFP from a PGK promoter (*Tiscornia et al., 2006*). Briefly, trophoblast cells were grown on 12-well plates until cultures reached 70% confluence. Lentiviral particles were added to the culture at a multiplicity of infection (MOI) of 10. Trophoblast cells with the highest GFP levels were collected by fluorescence-activated cell sorting (FACS) and cultured further. To generate spheroids, suspensions of GFP+ JEG-3 cells were adjusted to a 3×10^4 cells/ml concentration; 100 µl were seeded in U-bottom ultra-low attachment 96-well plates (Corning, NY, USA) and centrifuged at room temperature for 10 min at 250*g*. The plates were kept for 48 h at 37°C and 5% CO_2, resulting in spheroids of around 250 µm diameter.

In-vitro co-culture assay

In order to analyse the transcriptional dynamics during the first 24 h of trophoblast–epithelium interaction, an in-vitro system modelling embryo attachment was established as previously described (*Vergaro et al., 2019*). Briefly, Ishikawa cells were used as the substrate (representing the receptive endometrial epithelium), while GFP+ JEG-3 spheroids represented the embryo trophoblast. The epithelial substrates were grown in 96-well plates (Nunc, Thermo Fisher

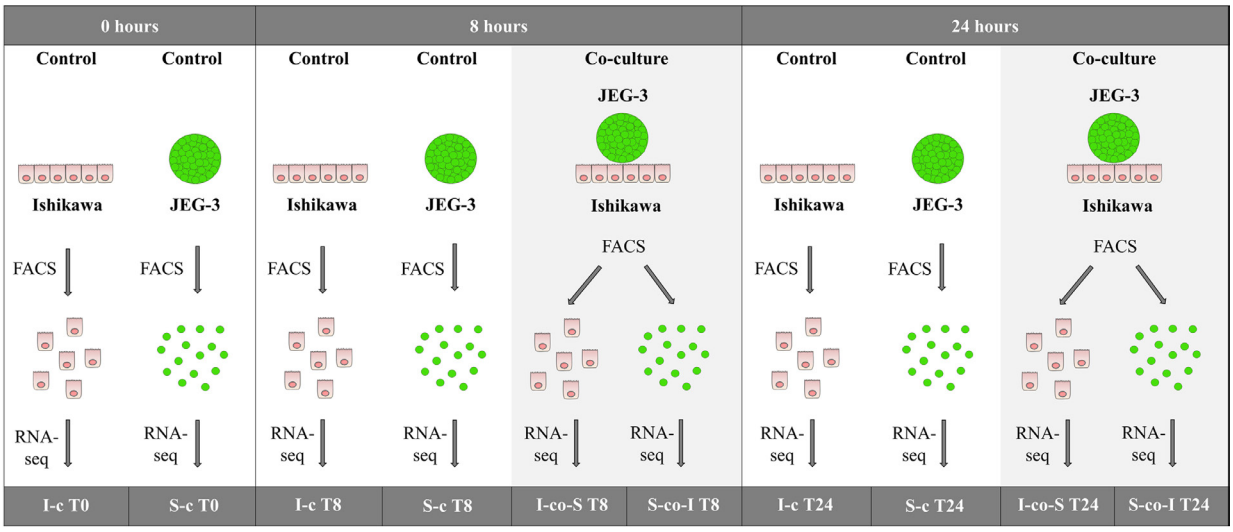

FIGURE 1 Scheme representing the experimental design: monolayers of receptive epithelium (Ishikawa) were co-cultured with trophoblast spheroids (JEG-3) for 0, 8 and 24 h, or cultured alone for the same time intervals as controls. Likewise, single spheroids were co-cultured with Ishikawa monolayers for 0, 8 and 24 h or cultured for the same time intervals in absence of any substrate as controls. All the JEG-3 spheroids expressed green fluorescent protein (GFP). After 0, 8 and 24 h, GFP+ (trophoblast) and GFP– (receptive epithelia) were separated by fluorescence-activated cell sorting (FACS) and analysed by RNA-seq. The transcriptomic profiles of the following cell populations were obtained: Ishikawa control at 0 h (I-c T0), GFP+ JEG-3 spheroids control at 0 h (S-c T0), Ishikawa control at 8 h (I-c T8), GFP+ JEG-3 spheroids control at 8 h (S-c T8), Ishikawa substrates co-cultured with JEG-3 spheroids for 8 h (I-co-S T8), GFP+ JEG-3 spheroids co-cultured with Ishikawa substrates for 8 h (S-co-I T8), Ishikawa control at 24 h (I-c T24), GFP+ JEG-3 spheroids control at 24 h (S-c T24), Ishikawa substrates co-cultured with JEG-3 spheroids for 24 h (I-co-S T24) and GFP+ JEG-3 spheroids co-cultured with Ishikawa substrates for 8 h (S-co-I T24).

Scientific, Waltham, Massachusetts, USA) until cells reached confluence; trophoblast spheroids were individually seeded on each well on top of a confluent epithelial substrate. In parallel to co-cultures, 96-well plates with confluent Ishikawa cells only or U-bottom ultra-low attachment 96-well plates with trophoblast spheroids only were kept in culture as experimental controls. At time points 0, 8 and 24 h, the co-cultures and controls were harvested from the 96-well plates using TrypLE™ Express Enzyme and pooled in single cell suspensions for cell sorting (FIGURE 1). All reagents were purchased from Gibco, Thermo Fisher Scientific, Waltham, Massachusetts, USA. In-vitro co-culture assays were repeated in triplicate as independent experiments.

Cell sorting and RNA extraction

After co-culture, FACS (BD FACS Aria Fusion II cell sorter, BD Biosciences, Franklin Lakes, New Jersey, USA) was used to separate GFP+ trophoblast cells from non-fluorescent epithelial substrates from each co-culture at the different time points (0, 8 and 24 h). Cell suspensions were maintained on ice and the cytometer chamber was cooled to 4°C before cell sorting. To eliminate possible cellular aggregates, cell suspensions were filtered through a 70 µm mesh before sorting and 100 µm sorter nozzles were used to minimize clog formation. Diamidino-2-phenylindole (DAPI) staining allowed exclusion of dead cells from the isolated cell populations. GFP+ and GFP– populations were collected in 50 µl of DPBS without calcium or magnesium and kept on ice. After centrifugation, cell pellets were stored at –80°C until processing. In each experimental replicate, 10 cell populations were obtained (a total of 30 cell populations). RNA from each of the 30 populations (10 populations from three independent experiments) was isolated using RNeasy Mini Kit (QIAGEN B.V., Venlo, the Netherlands) following the manufacturer's instructions. RNA was eluted in 30 µl nuclease-free water and purified using the RNase-Free DNase Set (Qiagen). RNA concentration was measured by fluorometric quantitation (QuBit, Thermo Fisher Scientific, USA) and high RNA integrity number (RIN) was determined by the Bioanalyser 2100 System (Agilent Technologies, Santa Clara, California, USA), ranging from 8.6 to 10 in all samples.

cDNA library preparation and RNA-seq

A total of 30 RNA samples (three experimental replicates of each of the 10 cell populations sorted by FACS) were used for cDNA library preparation and subjected to RNA-seq analysis. All 30 samples were subjected to quality control before polyA mRNA purification using the NEBNext Poly(A) mRNA Magnetic Isolation Module (New England Biolabs, Ipswich, Massachusetts, USA) according to the manufacturer's instructions. cDNA synthesis, end repair and ligate adaptor for Illumina were performed using the NEBNext Ultra II RNA Library Prep for Illumina kit (New England Biolabs, Ipswich, Massachusetts, USA), following the manufacturer's instructions. The minimum number of required cycles was established by amplifying samples with SYBR Green. Each sample was labelled with a specific barcode during the amplification by the NEBNext Multiplex Oligos kit for Illumina (Index Primers Set 1). Three equimolar pools of 10 samples were generated; each pool was sequenced in a single 50 nt single read lane of an Illumina HiSeq 2500 Sequencing System, to obtain over 30 million reads per sample.

RNA-seq data analysis

Sequence reads were aligned to the hg19 (GRCh37) version of the human genome using STAR software v2.3.0e (*Dobin et al., 2013*) with default parameters. Once aligned, reads were binarized, sorted with sambamba v0.5.9 (http://lomereiter.github.io/sambamba/) and

imported to R (https://www.R-project.org/) with the inbuilt annotation in the Rsubread package (*Liao et al., 2013*). Biomart (*Smedley et al., 2015*) was used as a source for further annotations using the corresponding R package. Taking into account processing batch as covariate, differential expression was analysed with DESeq2 (*Love et al., 2014*). Pairwise comparisons between the different transcriptional profiles were analysed according to two time stages: stage I (from 0 to 8 h) and stage II (from 8 to 24 h).

Functional analysis

The pre-ranked version of gene set enrichment analysis (GSEA) was used to assess pathway enrichment (*Subramanian et al., 2005*) and applied to the ranking defined by the log2 fold change (log2FC) of the differential expression analysis using DESeq2, considering all the transcripts with adjusted *P*-value <0.05. Significantly enriched pathways were selected with an FDR cut-off at 10%. Pathway analyses were based on the Hallmark collection (*Liberzon et al., 2015*) after retrieval from the MsigDB (*Liberzon et al., 2011*). For visualization of Broad Hallmark gene sets, plots were generated with Circos version 0.67 (*Krzywinski et al., 2009*).

DEG analysis

Differential gene expression between the cell populations collected after FACS was analysed by linear regression and pairwise comparison. Cut-off for statistical significance was set at absolute log2FC ≥1 and adjusted *P*-value <0.05.

RNA-seq validation by quantitative polymerase chain reaction (qPCR)

RNA-seq results were validated by qPCR using 5 ng (in triplicates) of the same cDNA libraries previously sequenced. The expressions of selected genes (*CYP19A1*, *SPDYC*, *CASC1*, *TGFA*, *PROM1* and *SLC30A2*) were quantified in a final volume of 20 µl using 2x SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) on a CFX Real-Time PCR platform (Bio-Rad). The qPCR program included a denaturalization step for 30 s at 95°C followed by 40 cycles at 95°C for 5 s and at 60°C for 30 s. Forward and reverse primer sequences (5′ to 3′) were the following: GTGGACGTGTTGACCCTTCT and CACGATAGCACTTTCGTCCA for *CYP19A1*, TCAGCCTTCTGGAG GACAGT and CACCATGGCCAGGAG ATACT for *SPDYC*, GGTGGGATGC TGAAGGTAAA and AAAGGTGTCCAG GCTGAATG for *CASC1*, TTCCCAC ACTCAGTTCTGCTT and ACGTACCCAGAATGGCAGAC for *TGFA*, GCCACCGCTCTAGATACTGC and GCTTTTCCTATGCCAAACCA for *PROM1* and TTATGCAGAGCAT GGGTGTC and GAAGGTGCAGAT GGGGTCTA for *SLC30A2*. *PPIB*, *MAP4K4* and *GUSB* were selected as the most stable housekeeping genes using the Integrated Cotton EST Database (*Xie et al., 2011*); forward and reverse primer sequences (5′ to 3′) were the following: CATGTGGTGTTTGGCAAAGT and TTTATCCCGGCTGTCTGTCT for *PPIB*, CTTGGATGGTGTGTTCATGC and AGACCGAACAGAGGCAAAGA for *MAP4K4* and AAACGATTGCAG GGTTTCAC and CTCTCGTCG GTGACTGTTCA for *GUSB*. Analysis of melting curves showed single peaks for all the qPCR products. Relative gene expression data were calculated as the ratio between the gene expression values of the selected genes and the geometric average of *PPIB*, *MAP4K4* and *GUSB* expressions using the ΔΔCq method (*Pfaffl, 2001*; *Vandesompele et al., 2002*). Student's *t*-test was used to calculate a two-tailed *P*-value of the gene expression differences among the following pairwise comparisons: I-co-S T8 versus I-c T0 for *CYP19A1* and *CASC1*, I-co-S T24 versus I-co-S T8 for *SPDYC* and S-co-I T8 versus S-c T0 for *TGFA*, *PROM1* and *SLC30A2*. Cut-off for statistical significance was set at log2FC ≥1 and *P*-value <0.05.

RESULTS

Trophoblast spheroids started apposing to Ishikawa monolayers within around 3 h and remained in the same position after moving plates in circular paths at a speed of one rotation per second for approximately 10 s (*Garrido-Gómez et al., 2012*). In order to maximize the detection of transcriptional differences in both compartments, a first co-culture time of 8 h was set, when all 96 trophoblast spheroids in 96-well plates were firmly attached. Flattening and initial outgrowth were observed around 10–12 h. By 24 h, all spheroids were attached and exhibited flattened shape and outgrowth, as shown in FIGURE 2. The outgrowth was seen as invasion into the Ishikawa cell layer by inverted microscopy, as trophoblast cells displaced Ishikawa cells.

The cell populations were isolated by FACS at different time points (0, 8 and 24 h) and analysed by RNA-seq profiling. Each co-culture gave rise to a GFP positive (JEG-3 trophoblast spheroids) and a GFP negative (Ishikawa epithelial substrates) cell population; control samples consisting of both cell types cultured in isolation were also independently sorted. A total of 10 gene expression datasets were obtained (Supplementary Table 1). Pairwise comparisons of co-cultured cells and their respective controls (culture in isolation) were used to analyse the effect of the trophoblast–epithelium interaction on the compartment-specific transcriptional profile over time, divided into two stages of co-culture: stage I (from 0 to 8 h) and stage II (from 8 to 24 h). To analyse the effect of the co-culture of each compartment on the transcriptional profile of the other compartment over time, the transcriptomic profile of populations co-cultured for 8 h was compared with the transcriptomic profile at 0 h. Similarly, the transcriptomic profile at 24 h was compared with that at 8 h. RNA-seq results are available in the Gene Expression Omnibus (GEO) repository (accession GSE132524).

Principal component analysis

Principal component analysis (PCA) was used as quality control to identify technical bias between the three independent experiments (FIGURE 3). PCA revealed concordant clustering among sample groups and experimental triplicates; the first component (PC1) explained 87.05% of the sample variability.

Gene expression dynamics of the epithelial substrates induced by co-culture with trophoblast spheroids for 8 and 24 h

Differential gene expression analysis of non-co-cultured epithelial substrates during stage I and stage II were expected to reflect any effect of the time of culture on the epithelium transcriptome; no genes were differentially expressed in non-co-cultured epithelial controls during stage I (I-c T8 versus I-c T0) or stage II of culture (I-c T24 versus I-c T8), indicating that the substrates were transcriptionally stable over these timeframes. Co-culture during stage I (I-co-S T8 versus I-c T0) resulted in differential expression of 295 genes in the epithelial substrates (200 genes up-regulated and 95 down-regulated) (FIGURE 4A; Supplementary File 1). Co-culture during stage II

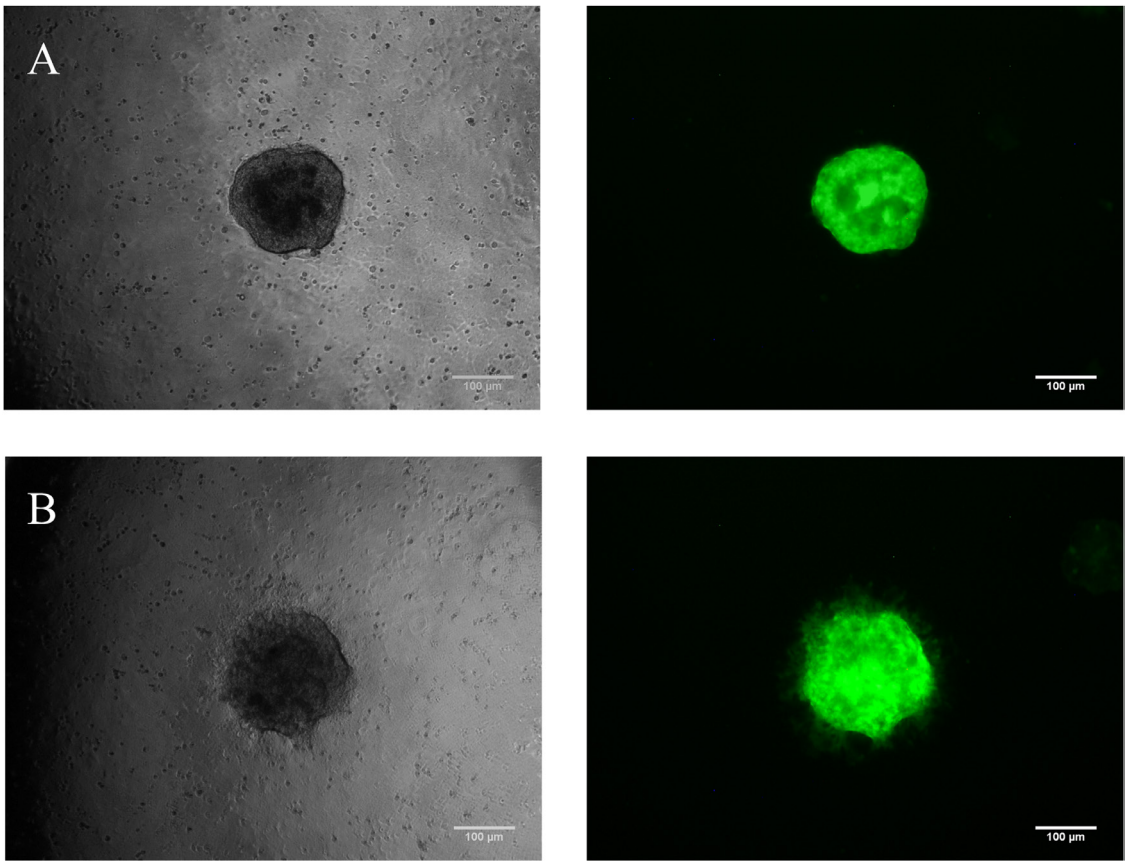

FIGURE 2 Representative images of GFP+ trophoblast spheroids co-cultured with Ishikawa monolayers for (A) 8 h and (B) 24 h.

(I-co-S T24 versus I-co-S T8) resulted in differential expression of 258 genes, of which 127 genes were up-regulated and 131 genes were down-regulated (FIGURE 4A; Supplementary File 2). A total of 124 genes were differentially regulated in both stages. Interestingly, 84 out of the 124 genes were up-regulated during stage I (I-co-S T8 versus I-c T0) and down-regulated during stage II (I-co-S T24 versus I-co-S T8), indicating an early wave of gene expression. Thirty-four genes were down-regulated during stage I and subsequently up-regulated in stage II, and six genes were up-regulated during both stages (FIGURE 4C; Supplementary File 3).

GSEA of the epithelial substrates

GSEA was performed using Broad Hallmarks annotation to identify the biological pathways enriched in the epithelial substrates during both stages of co-culture, illustrated in FIGURE 5 and presented in Supplementary Tables 2 to **5**. During the early interaction with the trophoblast (stage I), the enriched pathways were related to epithelial to mesenchymal transition (EMT), endocrine response (e.g. oestrogen response early), cell cycle (e.g. apoptosis, MYC targets v1, MYC targets v2), morphogenesis (e.g. myogenesis), cell signalling (e.g. regulation of the JAK-STAT, TGF, KRAS and TNF signalling cascades) and immune response (e.g. inflammatory response and allograft rejection). Strikingly, many of the pathways over-represented in stage I were found under-represented in stage II of culture (from 8 to 24 h).

Trophoblast spheroid gene expression dynamics induced by co-culture with epithelial substrates for 8 and 24 h

Similar to what was observed for the substrate, no genes were differentially expressed in the non-co-cultured trophoblast spheroid control during stage I (S-c T8 versus S-c T0). Subsequently, only six genes changed their expression levels during stage II (S-c T24 versus S-c T8); of these, four were up-regulated (*PLAC8*, *NRN1*, *IL2RB* and *RHPN1*), while two were down-regulated (*CES1* and *STX11*).

The variations in trophoblast cell gene expression levels in response to co-culture were numerous. During stage I, spheroids co-cultured with the epithelial substrate (S-co-I T8 versus S-co-I T0) showed differential expression of 1247 genes, of which 1201 and 46 genes were up- and down-regulated, respectively (FIGURE 4B; Supplementary File 4). During stage II, 481 genes were differentially expressed, of which 458 genes were up-regulated and 23 genes were down-regulated (S-co-I T24 versus S-co-I T8; FIGURE 4B; Supplementary File 5).

Comparing the differentially expressed genes in both stages of co-culture, 260 genes were found to be common between these timeframes (S-co-I T8 versus S-c T0 and S-co-I T24 versus S-co-I T8); most of these genes were persistently up-regulated at both 8 versus 0 h and at 24 versus 8 h (252/260). Of the remaining eight genes, four of them were up-regulated in stage I and down-regulated in stage II, while four genes were down-regulated at both stages (FIGURE 4D; Supplementary File 6).

GSEA of the trophoblast spheroids

The trophoblast spheroids were subjected to a GSEA analysis similar to that performed on the epithelial compartment. Significant Broad Hallmarks during both

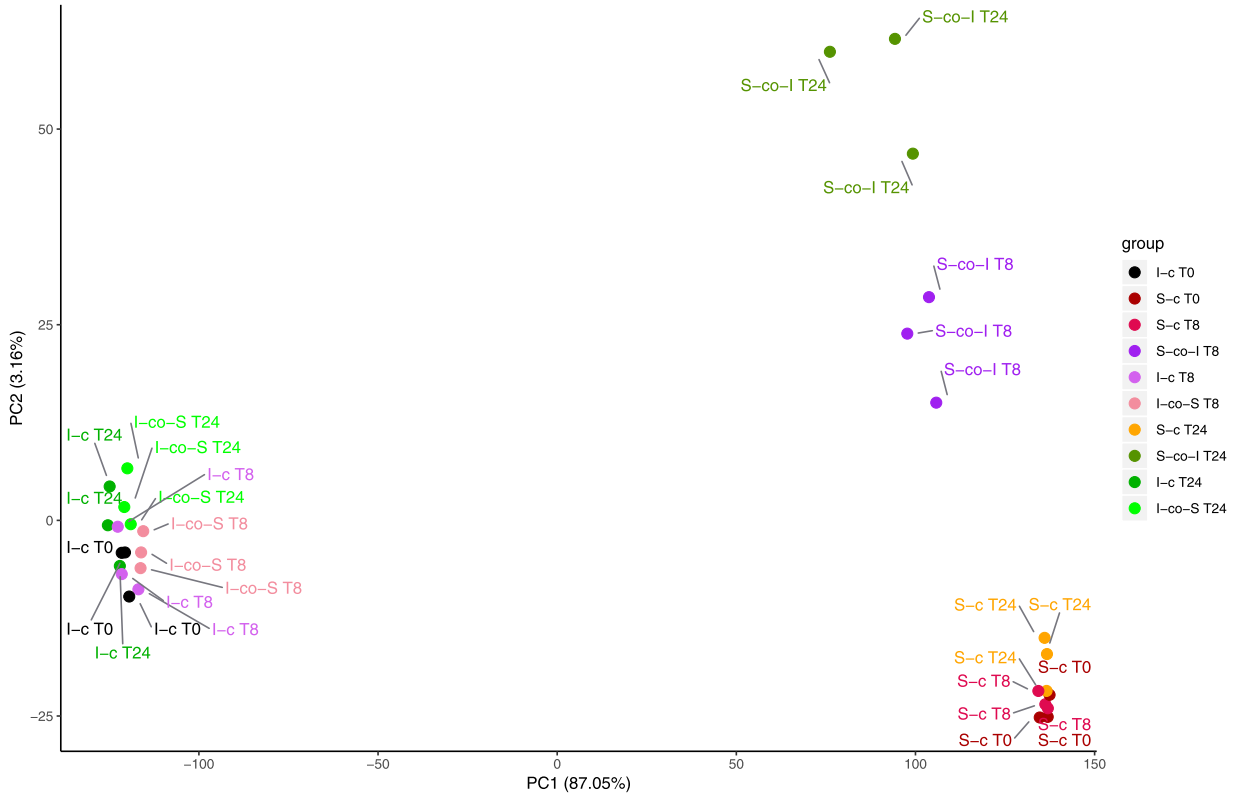

FIGURE 3 Principal component analysis representing all samples from the three experimental replicates according to principal component 1 (PC1) and principal component 2 (PC2): Ishikawa control at 0 h (I-c T0), JEG-3 spheroid control at 0 h (S-c T0), Ishikawa control at 8 h (I-c T8), JEG-3 spheroid control at 8 h (S-c T8), Ishikawa substrates co-cultured with JEG-3 spheroids for 8 h (I-co-S T8), JEG-3 spheroids co-cultured with Ishikawa substrates for 8 h (S-co-I T8), Ishikawa control at 24 h (I-c T24), JEG-3 spheroid control at 24 h (S-c T24), Ishikawa substrates co-cultured with JEG-3 spheroids for 24 h (I-co-S T24) and JEG-3 spheroids co-cultured with Ishikawa substrates for 24 h (S-co-I T24).

stages are illustrated in FIGURE 6D. During stage I, EMT, angiogenesis, signalling (e.g. KRAS signalling up and Wnt/β CATENIN signalling) as well as hypoxia were enriched; by contrast, pathways related to protein processing (e.g. unfolded protein response) and proliferation (e.g. MYC targets v2) were under-represented. At 24 h of co-culture, enriched pathways included hypoxia and angiogenesis as well as inflammatory and oestrogen responses. Conversely, cell proliferation pathways (e.g. E2F targets, G2M checkpoint and MYC targets v1 and v2) were under-represented.

RNA-seq validation

The RNA-seq results were validated by confirming gene expression patterns of up- and down-regulation of selected candidates. As in the RNA-seq data, *CYP19A1*, *TGFA*, *SPDYC* and *PROM1* were up-regulated, while *CASC1* and *SLC30A2* were down-regulated (FIGURE 7).

DISCUSSION

Surprisingly, only a few studies have used high-throughput techniques to analyse human implantation (*Huang et al., 2018*; *Moreno-Moya et al., 2015*; *Popovici et al., 2006*). In this work, GFP+ trophoblast spheroids were co-cultured with GFP– receptive epithelial cells, separated by FACS, and the transcriptomic response of each element of the model to the other was analysed during the early and late phases of apposition and attachment. Both the trophoblast and the endometrial epithelium showed a highly dynamic response, with marked transcriptional differences between 0 to 8 h and 8 to 24 h of co-culture.

Broad Hallmarks analysis yielded relatively short lists of pathways with relatively high normalized enrichment scores (NES), and many of them were found to be consistent with previous published reports. Data from this study suggest that co-culture of both compartments induced a transient wave of transcriptional up-regulation in the endometrial epithelium during the first 8 h, with many of the pathways enriched during stage I subsequently being under-represented in stage II. The most highly differentially expressed gene in stage I was *CYP19A1*, which has been shown to be involved in decidualization as well as increased susceptibility to unexplained female infertility and endometriosis (*Altmae et al., 2009*; *Gibson et al., 2013*). Several genes such as *LIF* and *CD44*, previously related to implantation success, showed an interesting pattern of expression with up-regulation during stage I and down-regulation in stage II. *LIF* is crucial for mouse embryo implantation and a marker of endometrial receptivity in fertile women (*Aghajanova, 2010*; *Cheng et al., 2001*; *Stewart et al., 1992*). Although its role in infertility is controversial, recent findings suggest that repressing *LIF* expression inhibits embryo attachment (*Huang et al., 2018*). In a recent report, functional blocking of epithelial CD44 led to delayed attachment during early stages (*Berneau et al., 2019*). Other differentially expressed genes are considered endometrium-specific (*Uhlen et al., 2015*), including *CPXM1*, *SOX17* and *ZCCHC12* (all up-regulated in stage I), and *TMEM158* (up-regulated in stage I and down-regulated in stage II). *CPXM1* has been associated with early-onset pre-eclampsia (*Song et al., 2015*); *TMEM158* gene expression has been found down-

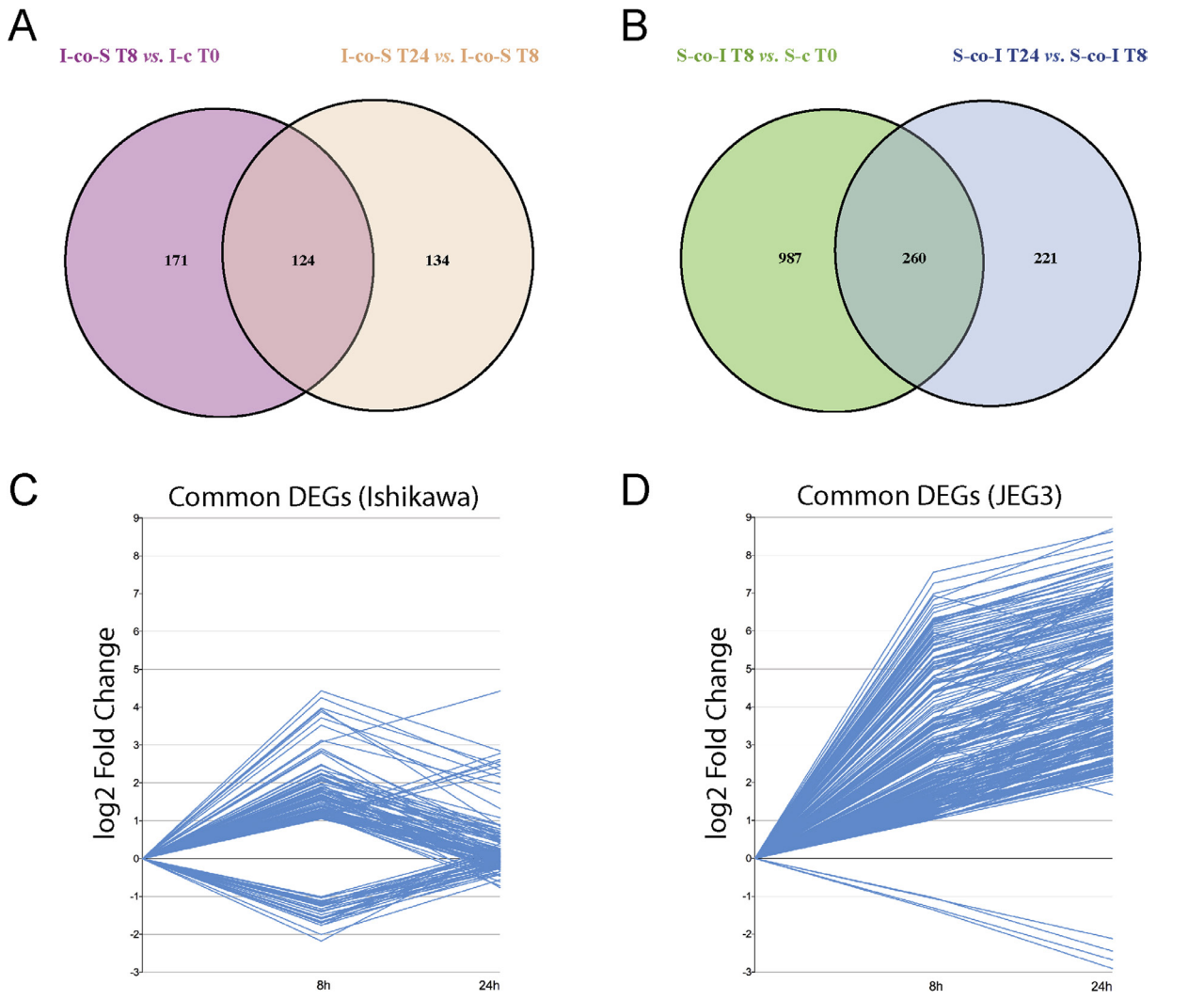

FIGURE 4 (A) Venn diagram representing differentially expressed genes (DEG) between the following pairwise comparisons: Ishikawa substrates co-cultured with JEG-3 spheroids for 8 h versus Ishikawa control at 0 h (I-co-S T8 versus I-c T0), and Ishikawa substrates co-cultured with JEG-3 spheroids for 24 h versus Ishikawa substrates co-cultured with JEG-3 spheroids for 8 h (I-co-S T24 versus I-co-S T8). (B) Venn diagram representing DEG between the following pairwise comparisons: JEG-3 spheroids co-cultured with Ishikawa substrates for 8 h versus JEG-3 spheroid control at 0 h (S-co-I T8 versus S-c T0), and JEG-3 spheroids co-cultured with Ishikawa substrates for 24 h versus JEG-3 spheroids co-cultured with Ishikawa substrates for 8 h (S-co-I T24 versus S-co-I T8). (C) Graphical representation of the transcriptional dynamics of common genes differentially expressed in Ishikawa substrates at stage I (from 0 to 8 h) and stage II (from 8 to 24 h): I-co-S T8 versus I-c T0 and I-co-S T24 versus I-co-S T8. (D) Graphical representation of the transcriptional dynamics of common genes differentially expressed in JEG-3 spheroids at stage I (from 0 to 8 h) and stage II (from 8 to 24 h): S-co-I T8 versus S-c T0 and S-co-I T24 versus S-co-I T8.

regulated in the post-implantation luminal epithelium of mice compared with that on the preimplantation period (*Xiao et al., 2014*). *SOX17* has been involved in embryo attachment, because it is predominantly located at the luminal epithelium in mice and shows increased levels at the embryo attachment sites (*Hirate et al., 2016*; *Wallingford et al., 2013*); of note, *sox17* haploinsufficiency results in female subfertility and implantation failure in mice (*Hirate et al., 2016*). *ZCCHC12* encodes a transcriptional coactivator in the bone morphogenetic protein (BMP)-signalling pathway (*Cho et al., 2008*). Although a role in implantation has not been reported, *ZCCHC12* has been identified as a marker of endometrial receptivity (*Hu et al., 2014*).

CEMIP (hyaluronic acid binding protein), which positively regulates cell migration by increasing EMT (*Liang et al., 2018*), was down-regulated during stage II (8 to 24 h of co-culture), in accordance with the negative enrichment of biological pathways related to cell motility and EMT. Another interesting candidate is PGR (progesterone receptor). The depletion of epithelial PGR expression levels is a marker of murine endometrial receptivity (*Wetendorf et al., 2017*) and a recent study found that FOXO1-PGR signalling is related to epithelial depolarization and tissue integrity (*Vasquez, 2018*). Data from this study showed that *PGR* expression did not change in stage I but was up-regulated during stage II, suggesting that after the initial trophoblast–epithelium interaction and barrier breaching, up-regulation of *PGR* is needed to initiate the reconstruction of the epithelial lining.

EMT has been suggested as a mechanism to reorganize the structure of the luminal epithelium (the first physical barrier that the embryo needs to breach

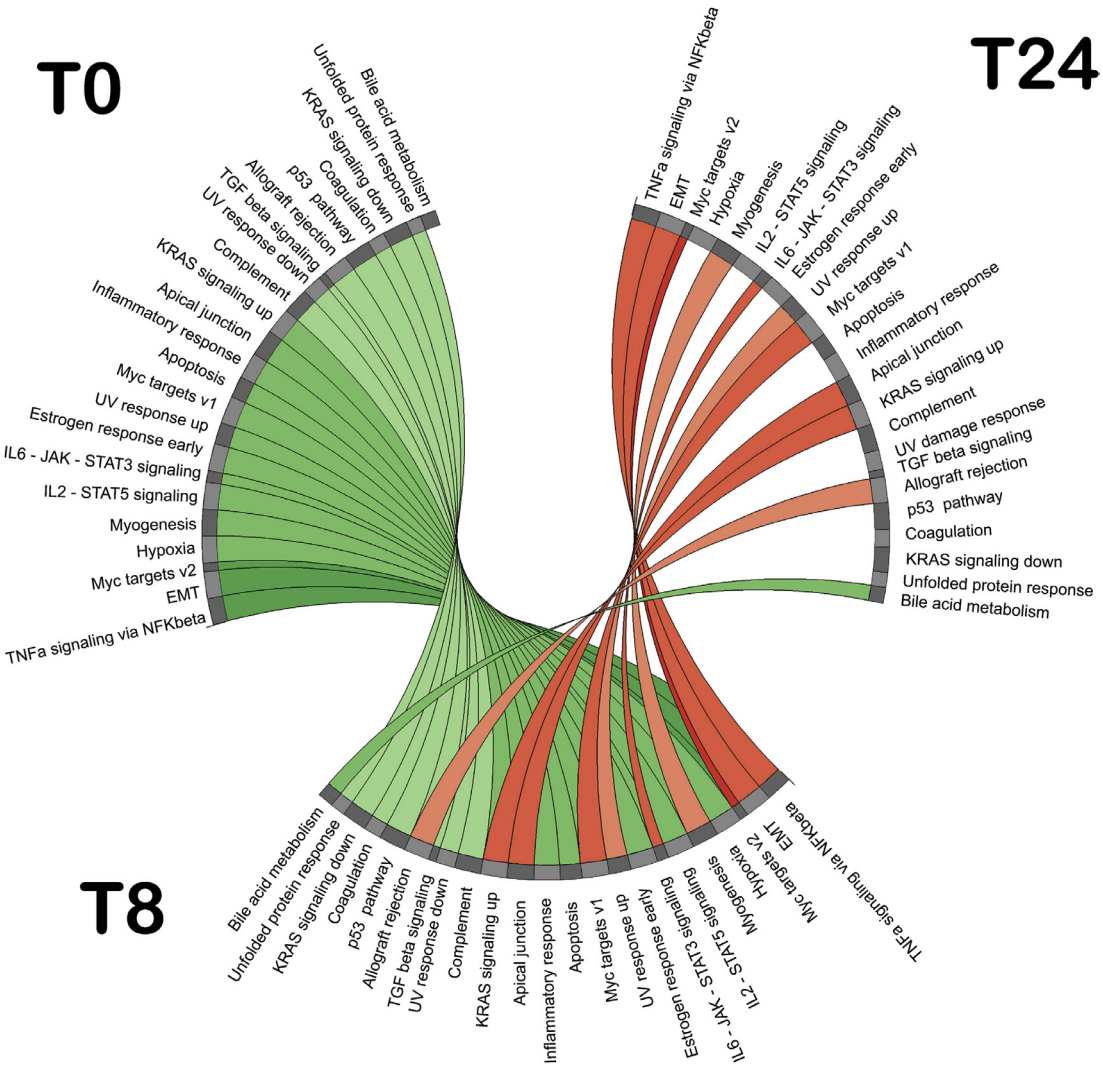

FIGURE 5 Graphical representation of the Broad Hallmark (BH) gene sets modulation in Ishikawa epithelial substrates co-cultured for 8 and 24 h with JEG-3 spheroids. Ishikawa non-co-cultured cells were used as T0. Ribbons are colour coded based on the normalized enrichment score (NES) of the pairwise comparison (very dark colours with |NES|>2, dark colours with 1.5<|NES|<2, and light colours with |NES|<1.5). Green and red represent enrichment or depletion of the BH gene set, respectively.

during implantation) in order to allow entry of the implanting trophoblast and then repair the breach (*Stone et al., 2016*; *Uchida et al., 2012*). EMT-related pathways were found as well as inflammatory and immune responses (i.e. TNFα signalling via NF-κB, IL6-JAK/STAT3 signalling and allograft rejection) were enriched in the substrate during stage I but under-represented as the co-culture progressed through stage II. This is consistent with increased levels of TNFα and IL-6 reported in women suffering from miscarriage and pathological pregnancies (*Banerjee et al., 2013*; *Lockwood et al., 2008*; *Quenby et al., 1999*); these molecules interact with IL-11, LIF and the JAK/STAT pathway, all of them involved in endometrial receptivity and implantation (*Dimitriadis et al., 2007*, *2010*; *Singh et al., 2011*). This could reflect the in-vivo event of EMT-mediated epithelial breaching followed by under-representation of EMT to rebuild the epithelial lining. Activation of other biological pathways, as suggested by this study, could also be involved in epithelial breaching, namely apical junction, actin filament-based movement and contraction and apoptosis (FIGURE 5), by promoting displacement of the cells on the luminal barrier. TGFβ signalling, known to up-regulate the expression of matrix metalloproteinases that promote trophoblast invasion (*Jones et al., 2006*), was also found enriched in stage I but not stage II.

The transcriptional response of the trophoblast spheroids followed a generally more dynamic pattern than that observed in the Ishikawa substrate; overall, it was characterized by early up-regulation during stage I that continued through stage II (of 260 genes differentially expressed at stages I and II, 252 were up-regulated at both stages). A number of genes have been shown to be involved in implantation, including *ERBB4* and *EPHA2* (proposed as markers for trophoblast proliferation and invasion (*Fock et al., 2015*; *Yang and Min, 2011*)), metalloproteinases (e.g. *MMP11* and *MMP17*) posited to contribute to trophoblast invasion during implantation and placentation (*Minas et al., 2005*; *Zhu et al., 2012*) and *TWIST1*, known to regulate trophoblast invasiveness via N-cadherin (*Ng et al., 2012*). Other genes (*PABPC4L*, *HOXA13*, *BIRC7* and

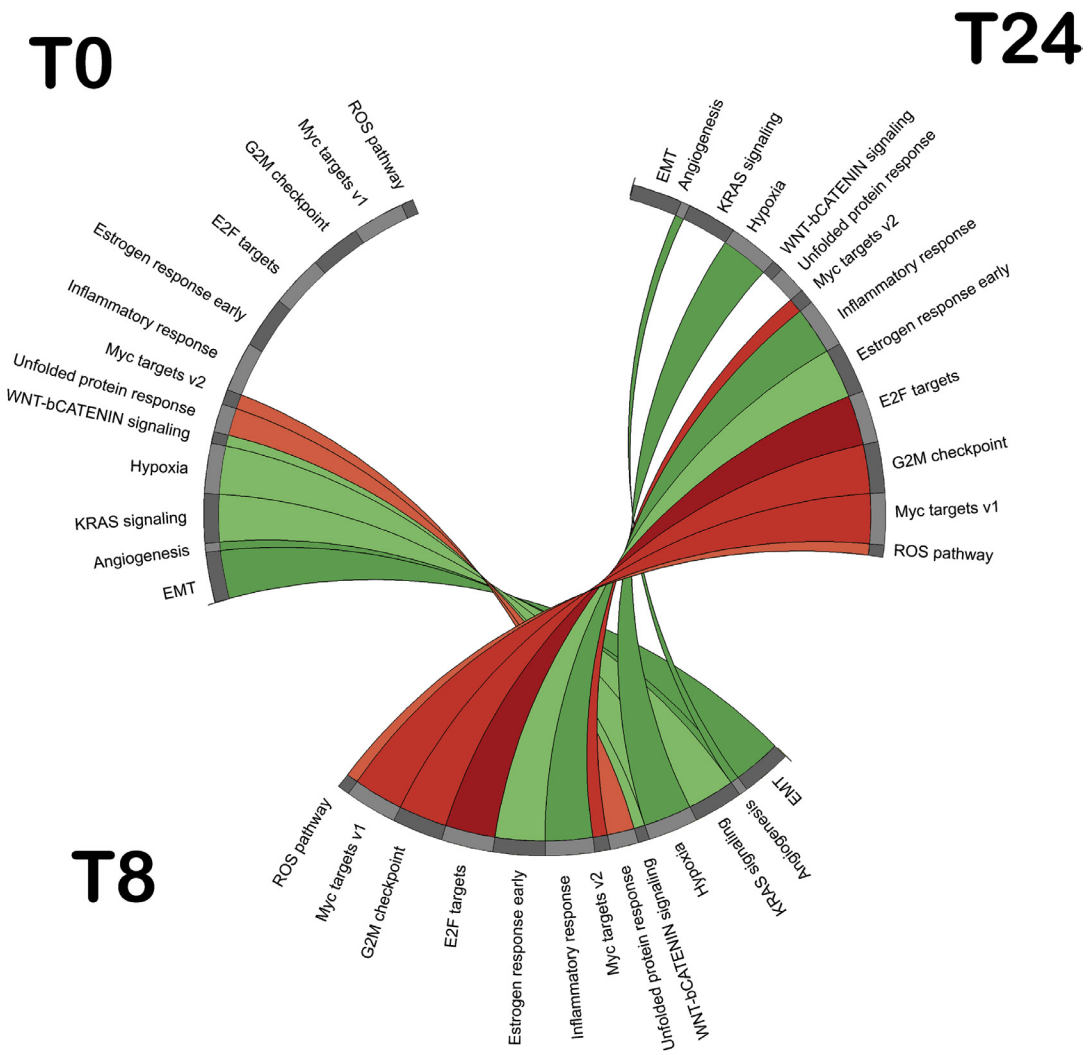

FIGURE 6 Graphical representation of the Broad Hallmark (BH) gene sets modulation in JEG-3 trophoblast spheroids co-cultured for 8 and 24 h on Ishikawa substrates. Non-co-cultured JEG3 spheroids were used as T0. Ribbons are colour coded based on the normalized enrichment score (NES) score of the pairwise comparison (very dark colours with |NES|>2, dark colours with 1.5<|NES|<2, and light colours with |NES|<1.5). Green and red represent enrichment or depletion of the BH gene set, respectively.

HES7) showing up-regulation at both stages are included in the Human Protein Atlas database as specific for placenta. Trophinin, another gene related to trophoblast invasion due to its dual action on promoting trophoblast proliferation and inducing endometrial epithelium apoptosis (*Sugihara et al., 2007*; *Tamura et al., 2011*) also showed the same pattern. Other placental-associated genes were only up-regulated in stage I (*ADAMTS15*, *IGFBP3* and *SPP1*) or in stage II (*JAM2* and *FLT1*). These molecules have been related to trophoblast proliferation and invasion (e.g. *IGFBP3*, *SPP1*, *BIRC* and *TIMP3*) (*Chen and Khalil, 2017*; *Gleeson et al., 2001*; *Li et al., 2014*; *Whiteside et al., 2001*; *Wu et al., 2015*), placental vascularization (e.g. *HOXA13*) (*Shaut et al., 2008*), attachment to the endometrial epithelium (e.g. *JAM2*) (*Su et al., 2012*) and angiogenesis (e.g. *FLT1*) (*Douglas et al., 2014*).

These data suggested a number of biological pathways previously related to trophoblast invasion and placental development, such as EMT, the Wnt/β-catenin pathway (canonical Wnt cascade), hypoxia and reduced proliferation (*Caniggia et al., 2000*; *Davies, 2016*; *Knofler and Pollheimer, 2013*; *Velicky et al., 2018*). Interestingly, trophoblast inflammatory response was up-regulated after 24 h but not before; it is postulated that, once the epithelium is breached, inflammation could act as a regulatory mechanism to prevent excessive invasion. Interestingly, angiogenesis was continuously increased along the 24 h of co-culture; if extrapolated to the in-vivo situation, the transcriptional up-regulation during this early time could trigger signalling required for remodelling of the maternal vasculature needed for placental development (*Kuo et al., 2019*; *Zhou et al., 2003*). Regulation of the unfolded protein response, which is linked to endoplasmic reticulum stress, has been related to placental development (*Burton and Yung, 2011*; *Yung et al., 2014*). Data from this study suggested a modulation of this system in the trophoblast compartment, with under-representation of unfolded protein response during stage I. Hypoxia (continuously enriched throughout both stages) and reactive oxygen species pathways (under-represented in stage I) might take part in this regulation.

In summary, the 2D in-vitro system in this study mimics early trophoblast

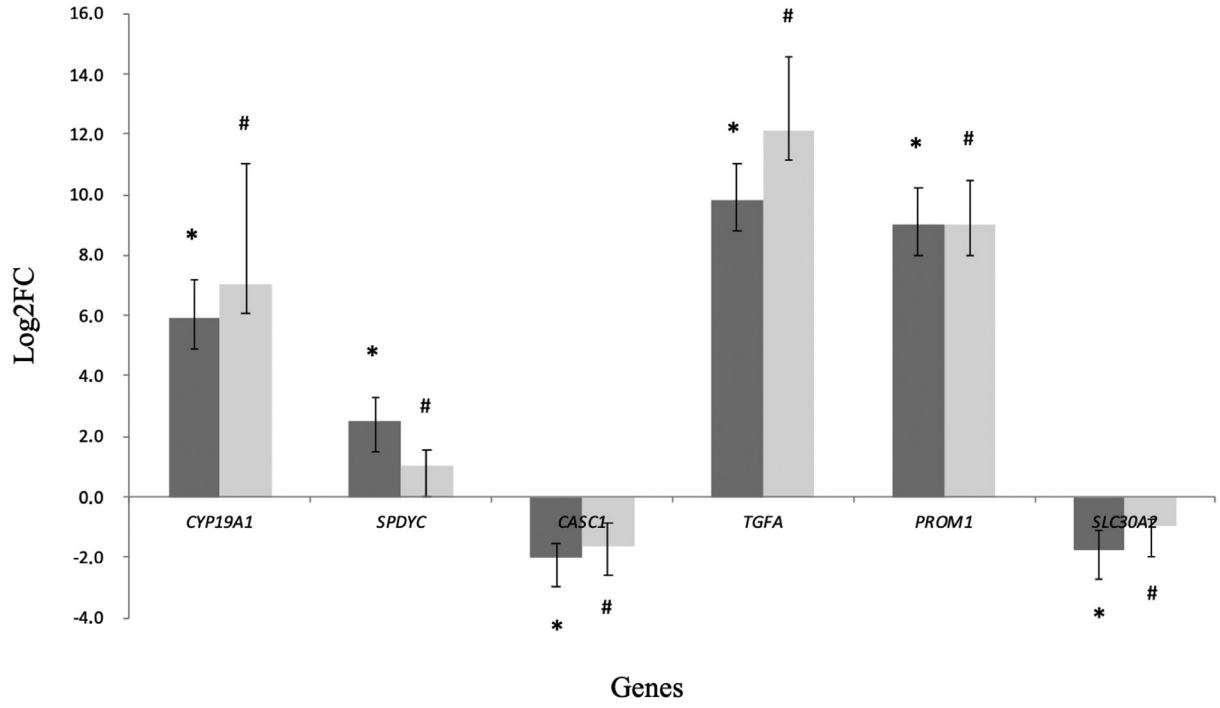

FIGURE 7 RNA-seq validation by qPCR. Dark grey bars represent the differences in gene expression levels among samples by RNA-seq and light grey bars represent the differences in gene expression levels among samples by qPCR. The genes were compared in the following samples: I-co-S T8 versus I-c T0 for *CYP19A1* and *CASC1*, I-co-S T24 versus I-co-S T8 for *SPDYC* and S-co-I T8 versus S-c T0 for *TGFA*, *PROM1* and *SLC30A2*. Asterisks and hash symbols indicate statistical significance of gene expression differences between samples by RNA-seq and qPCR, respectively ($P < 0.05$): *CYP19A1*, $P = 0.0148$; *SPDYC*, $P = 0.0174$; *CASC1*, $P = 0.0131$; *TGFA*, $P = 0.0012$; *PROM1*, $P = 0.0004$; *SLC30A2*, $P = 0.0168$. Error bars represent standard deviation among experimental values.

attachment and allows characterization of the transcriptional dynamics and molecular mechanisms regulating the process. The system has both drawbacks and advantages. Drawbacks are the carcinoma origin of the cell lines used, the lack of other elements present in the process (stromal, endothelial, and immune and stem cells) and certain characteristics of Ishikawa cells, such as the fact that despite their hormonal responsiveness *in vitro*, Ishikawa cells do not reflect the switch from non-receptive to receptive status driven by the steroid hormone levels *in vivo* (*Tamm-Rosenstein et al., 2013*); rather, Ishikawa cells are constitutively receptive to the trophoblast and hormonal supplementation during co-culture is not required (*Ruane et al., 2017*). This differs from the in-vivo situation and therefore caution is warranted when extrapolating these conclusions. Advantages are the practicality of using homogenous lines for this study (avoiding the cellular heterogeneity associated with primary endometrial tissue obtained from biopsies (*Suhorutshenko et al., 2018*)) and that the trophoblast JEG-3 cell line has a transcriptional profile and secretory activity similar to those from primary trophoblasts (*McConkey et al., 2016*). Likewise, Ishikawa cell line has been selected in many studies as a model for receptive epithelium and normal endometrial function (*Aberkane et al., 2018*; *Berger et al., 2015*; *Ruane et al., 2017*; *Schaefer et al., 2010*; *Singh et al., 2010*). Transcriptomic studies have shown Ishikawa is a good model to generate biologically relevant responses to hormonal and chemical treatments (*Naciff et al., 2016*).

A main advantage of the current approach is the use of GFP to separate and transcriptionally analyse both compartments individually. Compared with previous studies, this methodological advantage provided lists of compartment-specific candidate genes and suggested the need to focus not only on embryo quality and endometrial receptivity but also on their reciprocal molecular responses. Future confirmation in primary cells would be useful to find markers of implantation failure, with clinical application in both assisted conception and contraception. We believe this system would be appropriate to confirm a functional role of molecules previously related to implantation in a unilateral approach, and addition of other endometrial elements would add great value to the model (*Arnold et al., 2001*; *Brighton et al., 2017*; *Evron et al., 2011*; *Vento-Tormo et al., 2018*). In conclusion, the interaction between the trophoblast and the receptive epithelium preceding the establishment of pregnancy could rely on transcriptional programmes that are regulated in a space and time-specific manner. In the current model, this process is characterized by an early and transient transcriptional up-regulation in the receptive epithelium; in contrast, the transcriptional response of the trophoblast is less structured, with overall sustained up-regulation and involving different pathways at each stage.

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SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.rbmo.2020.08.037.

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